Western Blot

1. **Transfer Step**
    a. Transfer contents of SDS-Page gel to nitrocellulose membrane using western blot transfer apperatus
    b. Transfer Buffer (filter sterilize using 0.22 μm filter:
        - 20 mM Tris pH 8.0
        - 150 mM glycine
        - 30% v/v methanol
    c. Transfer for 1 hour at 250 Amps keeping the buffer cold with ice pack
2. **Blocking Step**
    a. Remove membrane from transfer apparatus
    b. Incubate the membrane in 25 mL blocking buffer, on rotating platform for 1 hour in the dark
    c. Blocking Buffer:
        - 25 mM Tris pH 8.0
        - 125 mM NaCl
        - 0.5% v/v Tween 20
        - 5% w/v skim milk powder
3. **Wash Step**
    a. Wash 2 times for 5 minutes in 25mM wash buffer, on rotating platform, in the dark
    b. Wash buffer:
        - 25 mM Tris pH 8.0
        - 125 mM NaCl
        - 0.5% v/v Tween 20
4. **Primary Antibody Incubation**
    a. Make a 1/30,000 dilution of the primary antibody (anti-RsaA) in 25 mL of blocking buffer
    b. Incubate the membrane the the mix, on rotating platform for 1 hour in the dark
5. **Wash Step**
    a. Wash 2 times for 5 minutes in 25mM wash buffer, on rotating platform, in the dark
6. **Secondary Antibody Incubation**
7. Make a 1/50,000 dilution of the secondary antibody (Goat-anti-rabbit w/ fluorophore) in 25 mL of blocking buffer
8. Incubate the membrane the the mix, on rotating platform for 1 hour in the dark
9. **Wash Step**
    a. Wash 2 times for 5 minutes in 25mM wash buffer, on rotating platform, in the dark
10. **Imaging**
    a. Use licor imager

b. Excite the fluorophore at 700 nm wavelength

Beta Carotene Extraction:

Materials Needed:

DH5α *E. coli* Cells having already expressed Beta Carotene
LB
Centrifuge
Antibiotics
Eppendorf tubes
Pipets

Protocol:

1. Grow 100ml of *E. coli* at 37C with induction of plasmid capable of expressing Beta-carotene at OD600 of ~0.4.
    a. If plasmid constitutively expresses beta-carotene do not induce.
2. After 24 hours of growing at 37C, pellet the cells by centrifugation at 4000rpm for 20 minutes.
    a. Make sure to take a measurement of the OD600 for use at a later time point.
3. Discard the supernatant and resuspend the cells in 5mL 100% acetone.
4. Heat in water bath at 50C for 10min to lyse the cells
5. Vortex the solution to homogenise, followed by centrifugation at 14,000 rpm for 1 minute
6. Collect supernatant and measure absorbance at 456nm.
    a. Normalize the absorbance measurement to cell density (OD600)

References

1. Adopted from iGEM Cambridge 2009 protocol for beta-carotene Isolation

Protocol for Restriction Enzyme Digest, Ligation, and Transformation:

1. Add 1ug of the plasmid to a new clean eppendorf tube and fill the tube with water until 42.5ul is reached. Keep enzymes on ice and add 1ul of each to both to the eppendorf tube. Add 0.5ul of BSA (if needed) and 5.0ul of the correct digestion buffer (a). flick the mixture to mix contents.
    a. Use Nebcutter to find correct buffer to use.
2. Incubate the digests at 37C for 60min in a water bath (a) or heat room (b).
    a. Ensure that the entire volume of solutions is under the water.
    b. Place it on a shaker or periodically mix the digest.
3. Incubate the three restriction digests at 80C in a water bath for 20min (a).
    a. Temperature need to heat inactivate enzymes is variable, in some cases 65C is enough. Check Nebcutter.
4. Refer to ligation calculator on the mixture you should use for the ligation (a,b) and then flick it to mix it.
    a. Keep T4 DNA ligase in a ice block to keep it around -20.
    b. The ligation buffer is located in -80C and put a dot on the container after using some of it.
5. Incubate the reaction mixture for 60-120min at room temperature, be sure to periodically flick the mixture to ensure it's mixed.
6. Heat inactivate the T4 DNA ligase by placing the mixture into a water bath at 80C for 20 min.
7. Obtain competent DH5alpha cells and thaw them on ice. Add ~2ul of the heat inactivated ligase mixture. Place the cells in 42C for 45secs and then return them back on ice for 2min. After, add 500-1000ul of lb and place the cells at 37C for 2 hours on a shaker.
8. Plate 150ul of cells onto the correct antibiotic.

PCR Master Mix

Making the Master Mix:

#PCR Tubes	1	2	3	4	5	6	7
10x Extaq Buffer	2.2	4.4	6.6	8.8	11	13.2	15.4
dH2O	12.65	25.3	37.95	50.6	63.25	75.9	88.55
dNTP	1.76	3.52	5.28	6.93	8.8	10.58	12.32
F Primer	0.44	0.88	1.32	1.76	2.2	2.64	3.08
R Primer	0.44	0.88	1.32	1.76	2.2	2.64	3.08
Polymerase	0.11	0.22	0.33	0.44	0.55	0.66	0.77
Template*	NA	NA	NA	NA	NA	NA	NA

*Template is dependent on the concentration of the thing you want amplified and should not be added to the master mix!

*The final volume of the template in the PCR tubes should be 4ul

- Be sure to keep the master mix on ice.
- Add the polymerase to the mix last.
- Add 16ul of the master mix to each PCR Tube for a net total of 20ul in the final PCR tube.
- Label all the PCR tubes different symbols, numbers, or letters and be sure to take a picture of the orientation of the tubes before you put them into the thermocycler as sometimes the Marker can be burnt off.
    - Ensure that the ALL PCR TUBES ARE CLOSED or things will evaporate

PCR Amplification

Master Mix for **3 reactions**:

10x Extaq Buffer	6.6 µL
dH2O	37.95 µL
2.5mM dNTP	5.28 µL
Forward Primer	1.32 µL
Reverse Primer	1.32 µL
Template	N/A
Polymerase	0.33 µL

1. Be sure everything above is taken from -20°C and is on ice, fully defrosted before use
2. Create master mix in an eppendorf tube-- add the polymerase last and do not spin once polymerase is added
3. Obtain PCR tubes-- label them all! (But make sure you remember in which order you place into the PCR machine because the marker does come off)
4. Pipet 4µL of water into the PCR tubes
5. Touch a colony with the pipet tip (to get biomass, but not a lot) and suck and release multiple times in the 4µL of water
6. Add 16µL of each master mix into each PCR tube and keep on ice
7. Take tubes and place in PCR Machine
8. Select iGEM folder and chose GA_16S for *G.apicola*, then click 'run'
9. After ~2 hours and 15 mins, open machine and take tubes out- place into the -20°C freezer
10. Turn off PCR machine

GA_16S, PCR Cycle for *G.apicola*

1. 10 min at 95°C
2. 30s at 94°C
3. 30s at 53°C
4. 1.5 min at 72°C
5. Cycle 34 times (steps 2-4)
6. 5 min at 72°C
7. infinity at 12°C

Colony PCR DNA Amplification:

Materials Needed:

PCR Mastermix Supplies
Thermocycler
PCR Tubes
1.5mL Eppendorf Tubes
Pipets
Cell Colonies/ DNA Template

Protocol (estimated time 2-6 hours):

1. Begin by removing all the PCR supplies from the -20°C freezer and thaw them on ice.
2. Fabricate the PCR master max in a 1.5mL eppendorf tube
    a. While making the mix, ensure the tube and its contents are kept on ice.
    b. Add the DNA polymerase (ExTaq) last
    c. Lightly spin the contents of the tube with your wrist prior to adding the extaq polymerase
    d. Depending on how many samples you need to run, refer to table 1 for master mix ingredients.

Table 1: PCR Master Mix							
#PCR Tubes	1	2	3	4	5	6	7
10x Extaq Buffer	2.2	4.4	6.6	8.8	11	13.2	15.4
dH2O	12.65	25.3	37.95	50.6	63.25	75.9	88.55
dNTP	1.76	3.52	5.28	6.93	8.8	10.58	12.32
F Primer	0.44	0.88	1.32	1.76	2.2	2.64	3.08
R Primer	0.44	0.88	1.32	1.76	2.2	2.64	3.08
Polymerase	0.11	0.22	0.33	0.44	0.55	0.66	0.77
Template*	NA	NA	NA	NA	NA	NA	NA

*Template is dependent on the concentration of the thing you want amplified and should not be added to the master mix if performing a colony PCR.

3. Obtain PCR tubes and label them all! (But make sure you remember in which order you place into the PCR machine because the marker does come off occasionally).
4. Pipet 4uL of water into each of the PCR tubes

5. Touch a colony with the pipet tip to get biomass, make sure there is not a lot. Rinse the pipet multiple times in the 4µL of water.
    a. Repeat for any amount of replicates or samples needed
    b. Please note, colony PCR can fail therefore we recommend doing at least 2 replicates for any high importance samples.
    c. To ensure there was no contamination in the PCR master mix, have a negative control with only master mix
    d. To ensure the PCR master mix works, have a positive control from the colony of the bacteria of interest
6. Add 16µL of the master mix into each PCR tube and keep on ice.
7. Take tubes and place them in the thermocycler. Set it up for the correct annealing temperature and elongation time.
    a. Before closing the machine lid, ensure all PCR tubes are closed. If they are open, even slightly, the samples inside will evaporate and become unusable.
    b. See Table 2.

Table 2: PCR Settings (1)		
Step 1	10 minutes at 95°C	Required
Step 2	30 seconds at 94°C	Required
Step 3	30 seconds at 53°C	Variable (primer annealing temperature dependent)
Step 4	1.5 minutes at 72°C	Variable (amplification base pair length dependent)
Step 5	Cycle steps 2 - 4 for 34 times	Variable (dependant on the amount of DNA wanted at end of amplification (2^x))
Step 6	5 minutes at 72°C	Required
Step 7	Infinity at 12°C	Required

8. After all steps are completed and the samples are at 12°C, turn off the thermocycler. Transfer all the tubes into an ice box for use in a PCR clean up, gel electrophoresis, or any other desired application.

References

1. Koch, H. et al. (2013). Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. *Molecular Ecology, 22(7)*. doi: 10.1111/mec.12209

DNA Agarose Gel Electrophoresis:

Materials Needed:

150mL 0.5X TAE Buffer
50mL 1X TAE Buffer
Horizons 58 Gel Electrophoresis Machine
Voltage Machine
5µL CyberSafe™
Balance
250mL Erlenmeyer Flask
Microwave
Parafilm
Graduated Cylinder
Pipets

Protocol (estimated time 1.5 hours):

1. Obtain a clean 250mL Erlenmeyer flask and add 500mg of agarose and 50mL 1X TAE buffer for a 1% agarose gel.
    a. Refer to Table 1 for percentage gel needed for optimum resolution

Table 1: Agarose Gel Percentage for Optimum Resolution		
Percent Agarose	mg agarose/100ml of 1X TAE Buffer	Optimum Resolution (kb)
0.5%	500mg	30 - 1.0kb
0.7%	700mg	12 - 0.8kb
1.0%	1000mg	10 - 0.5kb
1.2%	1200mg	7 - 0.4kb
1.5%	1500mg	3 - 0.2kb

2. Heat agarose in microwave for approximately 1 minute or until the solution starts to boil. Check to ensure that all the agarose has dissolved.
3. Swirl the Erlenmeyer flask under running cold water until the flask becomes warm to the touch.
4. Add 5µL of Cybersafe™ to the solution and swirl to ensure the Cybersafe™ has fully diffused.

5. Construct the gel casting station of the Horizons 58 Gel machine and pour in the agarose.
6. Remove all bubbles by using the comb or pipet tips.
    a. Add the comb to the black side of the machine (DNA will run from black to red).
    b. Let the gel sit for 20 minutes to solidify.
7. Remove the sides of the casting station and pour enough 0.5X TAE buffer so that the gel is completely covered on top. Remove the comb to expose the wells.
8. Obtain a small piece of parafilm and pipet 1µL of 6X DNA loading dye onto the parafilm film for every sample you need to run. Pipet 6µL of sample into the 1µL drops of loading dye and then pipet up all 7µL of sample and loading dye into the pipet and inject sample into well.
    a. Repeat for as many samples as you have (leaving one well open for latter).
9. Add 5µL of a DNA ladder to a well and close the machine lid.
10. Attach cables and turn on the voltage machine.
    a. Set machine to run at 100 Volts for 30-45 minutes (the time is dependant on the desired band separation).
11. After the 30-45 minutes, turn off the machine, unplug wires, and remove the gel.
12. View the bands on an AlphaImager™ or other imaging machine.

Chemically Competent E. coli Transformation:

Materials Needed:

Competent DH5α *E. coli* cells
Plasmid
LB
Water bath
LB with antibiotic plates
Eppendorf tubes
Pipets

Protocol:

1. Take competent DH5α *E. coli* cells from -80°C. In an Eppendorf tube, put 50μL for transforming a DNA construct or 100 μL for transforming a ligation. More or less cells are required depending on how competent the cells are. Keep the tubes on ice
2. Turn on water bath to 42°C
3. Add 1 μL of circular DNA into the *E. coli* cells.
4. Incubate on ice for 10 minutes
5. Place tube(s) with DNA and *E. coli* into the 42°C water bath for 45 seconds
6. Place tubes back on ice for 2 minutes to reduce damage to the *E. coli* cells
7. Add 1 mL of LB (with no antibiotic added) into each tube.
8. Incubate tubes for 30 minutes to 1 hour at 37°C, chose the latter if transforming a ligation
9. Spread about 100 μL of the resulting culture on LB plates (with appropriate antibiotic added) Grow overnight and pick colonies once grown.

References

1. Teruel, M. (2003). Transformation Protocol Using Heat Shock. Stanford databases. Retrieved from http://web.stanford.edu/~teruel1/Protocols/.

Caulobacter Electroporation Transformation:

Materials Needed:

- Electrocompetent Cells
- PYE Plates with Chloramphenicol (2 ug/mL)
- PYE Media
- Glass beads
- Pipets
- Electroporation Cuvettes
- Kimwipes
- Inoculation Loop

Protocol (estimated time 2-3 days):

1. Thaw your electrocompetent cells and plasmid on ice. Additionally, chill two electroporation cuvettes on ice.
    a. Cuvettes are placed on ice to prevent cell shock when transferred to a new environment
2. After about 20 minutes of chilling the electroporation cuvettes, pipet in 1µL of plasmid DNA (50-100µg) and 50µL of cells.
    a. The cells are either allowed to rest with the DNA for 15 - 30 minutes or are taken directly electroporated.
        i. Some protocols recommend resting with DNA, whereas others recommend electroporating right away.
3. Wipe down the sides of the electroporation cuvette with a kimwipe and place it into the electroporation machine.
    a. Wiping down the outside prevents sparks from forming/damaging the machine.
4. Electrocute the cells 1800 kV, 200ohms, 25µF with a time constant less than 4ms.
    a. No arcing should occur.
5. Gently remove the electroporation cuvette from the machine and pipet in 1000µL PYE media. Pipet the cuvette contents into eppendorf tube
6. Incubate tube, shaking, for 2 hours at 30℃
7. Repeat steps 4 through 6 with a no plasmid control
8. Spin down contents of eppendorf tube at 8000 rpm for 2 minutes
    a. Remove 900 µL of supernatant
    b. Resuspend the cell pellet in the remaining supernatant
9. Plate the cell suspension on a PYE plates and incubate for 2-3 days at 30℃
10. Once the bacteria are scraped off the plate and floating/suspended in the TSB, angle the plate to one side and pipet up all the TSB and cells
    a. If there is growth on the negative controls of electroporated cells without plasmid on the antibiotic plates, then the transformation was unsuccessful. This may either be due to contamination or a poor concentration of antibiotic.

References

1. Williams, P., Ketley, J., & Salmond, G. (Eds.). (1998). *Bacterial Pathogenesis*. London, UK: Academic Press.